Germline cytotoxic lymphocytes defective mutations in Chinese patients with lymphoma

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Received February 25, 2016; Accepted July 7, 2017

DOI: 10.3892/ol.2017.6898

Abstract. Certain patients with lymphoma may harbor mutations in perforin 1 (PRF1), unc-13 homolog D (UNC13D), syntaxin 11 (STX11), STXBP2 (syntaxin binding protein 2) or SH2 domain containing 1A (SH2D1A), which causes functional defects of cytotoxic lymphocytes. Data regarding the association between genetic defects and the development of lymphoma in Chinese patients are limited to date. In the present study, 90 patients with lymphoma were analyzed for UNC13D, PRF1, STXBP2, STX11, SH2D1A and X-linked inhibitor of apoptosis. Mutations were observed in 24 (26.67%) patients; 16 patients exhibited mutations in UNC13D, 7 exhibited PRF1 mutations, and 1 exhibited monoallelic mutation in STX11. UNC13D c.2588G>A/p.G863D mutation was detected in 9 patients (10.00%) and in 4/210 controls (1.90%). This mutation was predicted to be pathogenic and it predominantly existed in the Chinese population. These findings suggest that impaired cytotoxic machinery may represent a predisposing factor for the development of lymphoma. Furthermore, these data describe a distinct mutation spectrum in Chinese patients with lymphoma, whereby UNC13D is the most frequently mutated gene. In addition, these findings suggest UNC13D c.2588G>A mutation is a founder mutation in Chinese patients.

Introduction

The perforin-dependent granule-mediated cytolysis of cytotoxic lymphocytes (CLs), including natural killer cells and cytotoxic T lymphocytes, is the key machinery in the clearance of viral, and intracellular bacterial infections, as well as in the prevention of tumor development (1,2). The proteins encoded by perforin 1 (PRF1), unc-13 homolog D (UNC13D), syntaxin 11 (STX11), and STXBP2 (syntaxin binding protein 2) serve an essential role in this pathway. Mutations in these genes lead to function defects of CLs and are causative of familial hemophagocytic lymphohistiocytosis type 2 (FHL2), FHL3, FHL4, and FHL5 (3-6). The clinical manifestation of X-linked lymphoproliferative disease (XLP), which is caused by mutations in SH2 domain containing 1A (SH2D1A) (7) or X-linked inhibitor of apoptosis (XIAP) (8) genes, resembles hemophagocytic lymphohistiocytosis. Furthermore, XLP2 due to XIAP deficiency has been suggested to be classified as X-linked FHL (9).

A proportion of patients with lymphoma have been reported to harbor mutations in PRF1, UNC13D, STX11, STXBP2 or SH2D1A genes (10-14), indicating that genetic defective function of CLs may increase susceptibility to lymphomagenesis. The aim of the present study was to investigate the association between mutations in genes involved in the cytotoxic function of CLs and the development of lymphoma in Chinese patients.

Patients and methods

Cases and controls. In the present study, 68 and 34 patients with lymphoma were admitted to Hebei Yanda Lu Daopei Hospital (Sanhe, China) and Peking University First Hospital (Beijing, China), respectively, between August 2013 and August 2015; 12/102 were excluded due to poor DNA quality. A total of 90 (61 from Hebei Yanda Lu Daopei Hospital and 29 from Peking University First Hospital) unrelated patients with lymphoma (48 males and 42 females; age range, 3-60 years) were recruited in the present study; 39 were diagnosed with Hodgkin lymphoma and 51 were diagnosed with non-Hodgkin lymphoma according to the World Health Organization classification (15). Healthy donors of Han nationality (n=210) at the Hebei Yanda Lu Daopei Hospital served as controls. The present study was approved by the Ethics Committees of Hebei Yanda Lu Daopei Hospital and Peking University First Hospital. Written informed consent was obtained from all patients and healthy donors or their parents in accordance with the 1964 Helsinki declaration, and its later amendments or comparable ethical standards.

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Key words: cytotoxic lymphocytes, founder mutation, gene mutation, lymphoma

Amplification and sequence analysis. Genomic DNA was isolated from peripheral blood and bone marrow using the TIANamp Blood DNA kit (item no. DP318; Tiangen Biotech Co., Ltd., Beijing, China) or from nails using the TIANamp FFPE DNA kit (item no. DP331; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. Referenced coding sequences of the PRF1 (NM_005041.4), UNC13D (NM_199242.2), STXBP2 (NM_003764.3), STX11 (NM_006949.2), SH2D1A (NM_002351.3), and XIAP (NM_001167.2) were obtained from the National Center for Biotechnology Information Consensus CDS database (https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi). Primers were designed to amplify the coding exons and the flanking intron sequences by polymerase chain reaction (PCR). The sequences of primers are presented in Table I. The PCR system comprised of 1 μl genomic DNA (10 ng/μl), 1 ml forward primer (20 pmol/μl), 1 ml reverse primer (20 pmol/μl), 10 μl Phusion Flash High-Fidelity PCR Master mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 7 μl distilled water in a total volume of 20 μl. Reaction conditions were 10 sec at 98˚C followed by 38 cycles of 10 sec at 98˚C, 10 sec at 68˚C, 15 sec at 72˚C, and then 1 min at 72˚C. The amplified PCR products were purified with ExoSAP-IT (USB Co., Cleveland, OH, USA) and followed by cycle sequencing PCR using a BigDye Terminator Sequencing Kit version 3.1 (Thermo Fisher Scientific, Inc.). Fluorescent labeled products were separated using an ABI 3500xL Genetic Analyzer (Thermo Fisher Scientific, Inc.). Variations were analyzed using Variant Reporter software (version 1.1; Thermo Fisher Scientific, Inc.). Genetic polymorphism information from the Single Nucleotide Polymorphism database (dbSNP; http://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes Project (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) and the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) were referenced to obtain the frequencies of variants in large populations. Variants with minor allele frequencies >1% in the 1000 Genomes Project and/or ExAC were regarded as SNPs rather than mutations.

Confirmation of germline derivation of mutations. For patients determined to harbor mutations, the same mutation was detected in the DNA isolated from peripheral blood of their parents. In the absence of one or both parents, the detection of the same mutation in DNA extracted from nails of the patients could be of value. This was performed in order to confirm that the mutations were germline-derived.

In silico analysis. Two bioinformatics tools were used to predict whether an amino acid substitution was benign or deleterious: Sorting Intolerant From Tolerant (SIFT; http://sift.jcvi.org/) predicts whether an amino acid substitution affects protein function based on the degree of conservation of amino acid residues in multiple sequence alignments derived from closely associated sequences (16); and Polymorphism Phenotyping version 2.0 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph/) predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative analyses (17). Iterative Threading ASSEmbly Refinement (I-TASSER; http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was also used to predict and simulate the influence of the variants in protein tertiary structures.

Statistical analysis. Comparisons of mutant frequencies as well as genotype distributions between patients with lymphoma and controls were performed using the Chi-square test with SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of the gene mutations. A total of 18 different mutations were identified in 24 unrelated patients (26.67%) (Fig. 1). A total of 16 patients (17.78%) carried mutations in UNC13D, including 12 with monoallelic mutations, 1 with homozygous mutation and 3 with compound heterozygous mutations. Seven patients (7.78%) had PRF1 mutations, including 4 with monoallelic mutations, 1 with homozygous mutation and 2 with compound heterozygous mutations. One patient (1.11%) was detected to carry STX11 monoallelic mutation (Table II). All mutations were confirmed to be germline-derived.

Sixty unrelated healthy donors were sequenced for these 6 genes with the same methods and 5 of them (8.33%) were detected to harbor mutations. All 5 individuals were heterozygous for UNC13D mutations (c.680G>A/p.R227H; c.3134C>T/p.T1045M; c.3229_3235del/p.Arg1077SerfsTer48; c.2553+5C>G; c.602A>G/p.H201R).

The Chi-square test revealed that the difference between mutant frequencies of patients with lymphoma and healthy donors was of statistical significance ($P=0.005$). Individuals carrying mutations of these genes were more likely to develop lymphoma compared with those without mutations [odds ratio (OR), 4.000; 95% confidence interval (CI), 1.431-11.180].

Statistical analysis of UNC13D c.2588G>A mutation. UNC13D c.2588G>A/p.G863D was the most frequent mutation identified in the current study, which was identified in 9 patients (10.00%), including 1 homozygous and 8 heterozygous. This genetic variation was annotated as rs140184929 in dbSNP without frequency data. Data in the 1000 Genomes Project demonstrated that the c.2588A allele existed predominantly in the Chinese (0.83%), and rarely in the Japanese (0.48%) and Bengali (0.58%) populations. Other populations did not carry this variant (Table III). Data in ExAC also demonstrated that the allelic frequency of c.2588A was increased in East Asian populations (37/8,638; 0.43%) compared with that in South Asian populations (5/16,504; 0.03%). Only one individual out of 32,962 Europeans was heterozygous for c.2588G>A variant. This variation was not observed among 14,554 individuals analyzed from other populations. Considering the high allele frequency of this mutation in the present patient cohort and the distinctly different allele frequencies among diverse populations, genotyping of the c.2588 allele was performed in 210 unrelated healthy donors of Chinese Han nationality (Table III). Heterozygous c.2588G>A was observed in 4 of them. Combined with data in the 1000 Genomes Project (a total of 301 Chinese), a control cohort of 511 individuals, 9 of whom harbored c.2588A allele in a heterozygous state was obtained. The Chi-square test revealed that the allele frequency of c.2588A in patients was significantly increased compared with that in the control group ($P<0.001$; OR, 6.621; 95% CI, 2.652-16.532), suggesting an association between the c.2588G>A mutation, and the risk of developing lymphoma.

Table I. Primers used for amplification of the coding exons and the flanking intron sequences of perforin 1, unc-13 homolog D, syntaxin binding protein 2, syntaxin 11, SH2 domain containing 1A and X-linked inhibitor of apoptosis.

Name of the primer	Sequence 5' to 3'
UNC13D-1FS	**TGTAAAACGACGGCCAGT**ACTCGAGGAAGTGGGGTGAGA
UNC13D-1RS	*CAGGAAACAGCTATGACC*GAGACCACAGTGCTCCCCAA
UNC13D-2FS	**TGTAAAACGACGGCCAGT**CCTGTCCATCTGAGCCTGCTC
UNC13D-2RS	*CAGGAAACAGCTATGACC*GGGACCCCACCCCATGCTCA
UNC13D-3FS	**TGTAAAACGACGGCCAGT**GGTCAGGGAGCTTGAGGTAACC
UNC13D-3RS	*CAGGAAACAGCTATGACC*AGACCCTGCTACCCAGGAAAG
UNC13D-4FS	**TGTAAAACGACGGCCAGT**GCTCTGGGCTGTGGTCACTTAC
UNC13D-4RS	*CAGGAAACAGCTATGACC*AGGCTCAGCTTTGTGAGGACAC
UNC13D-5FS	**TGTAAAACGACGGCCAGT**CCTGGGGTCCACCTCCTGTC
UNC13D-5RS	*CAGGAAACAGCTATGACC*GCTGGTGGCTCAGGGGTTC
UNC13D-6FS	**TGTAAAACGACGGCCAGT**GGCAATTTCCTCCTCCCTGTC
UNC13D-6RS	*CAGGAAACAGCTATGACC*CAGTGGTGCCAGTCTGTCGAC
UNC13D-7FS	**TGTAAAACGACGGCCAGT**GCAGGGTCCTGGTACAGATGTG
UNC13D-7RS	*CAGGAAACAGCTATGACC*GCCATGGAGAAGAGGTGGATC
UNC13D-8FS	**TGTAAAACGACGGCCAGT**GGTGTATGCCACTGGGTGACA
UNC13D-8RS	*CAGGAAACAGCTATGACC*AGGTCCAGGCAGAACCCAAG
UNC13D-9FS	**TGTAAAACGACGGCCAGT**CTGGTGATGGTAGCTGCTCTATGA
UNC13D-9RS	*CAGGAAACAGCTATGACC*CAGCTGGGACAGAGATGCAGA
UNC13D-10FS	**TGTAAAACGACGGCCAGT**CCAGGCAGCCAACATGGTAA
UNC13D-10RS	*CAGGAAACAGCTATGACC*AGAGAACATGCTTTGCCTGGTC
UNC13D-11FS	**TGTAAAACGACGGCCAGT**CTACAAACTGCTCTCACAGAACGG
UNC13D-11RS	*CAGGAAACAGCTATGACC*GGCTGCTACACCCCTCAGAAC
UNC13D-12FS	**TGTAAAACGACGGCCAGT**GAGCGTCTTTGCTTCCTCCTC
UNC13D-12RS	*CAGGAAACAGCTATGACC*GCTCACTGTCAAGGGTAACATGTC
UNC13D-13FS	**TGTAAAACGACGGCCAGT**TCCCATGACCCAATACTTTCCA
UNC13D-13RS	*CAGGAAACAGCTATGACC*GCACTGACCCCTCCTGGTAAC
UNC13D-14FS	**TGTAAAACGACGGCCAGT**ACTCATCCGGAAGTACTTCTGCA
UNC13D-14RS	*CAGGAAACAGCTATGACC*CACATCCAGCTGCAAACTCTTG
UNC13D-15FS	**TGTAAAACGACGGCCAGT**AGCTGGCTTTGCAGTCCAAA
UNC13D-15RS	*CAGGAAACAGCTATGACC*TCAGACCGTTGCTGGTATCAAA
UNC13D-16FS	**TGTAAAACGACGGCCAGT**GGAGAAGGGCCTGGATCTCA
UNC13D-16RS	*CAGGAAACAGCTATGACC*CCTACAGGAAAGCCCTTGCA
STXBP2-1FS	**TGTAAAACGACGGCCAGT**GACTCAACTTCCTGGGCCTG
STXBP2-1RS	*CAGGAAACAGCTATGACC*GGAGCAGCTGAGGCCGGAACT
STXBP2-2FS	**TGTAAAACGACGGCCAGT**TGGTGGGACCAGAGAACCAG
STXBP2-2RS	*CAGGAAACAGCTATGACC*CACGCTCAGGTCCCATCTCA
STXBP2-3FS	**TGTAAAACGACGGCCAGT**TGGTGGTCCCTAAGTGGGTTTC
STXBP2-3RS	*CAGGAAACAGCTATGACC*GCATACACACACGCTCACTCATG
STXBP2-4FS	**TGTAAAACGACGGCCAGT**CCATGTGGGTGCGACACTAGT
STXBP2-4RS	*CAGGAAACAGCTATGACC*GCCCAGCCTCAGTGTCTGTTT
STXBP2-5FS	**TGTAAAACGACGGCCAGT**CAACCCTGGTGCTTCTGTCC
STXBP2-5RS	*CAGGAAACAGCTATGACC*GGAACCAGGTCAGTGGCAAG
STXBP2-6FS	**TGTAAAACGACGGCCAGT**CTTGCCACTGACCTGGTTCC
STXBP2-6RS	*CAGGAAACAGCTATGACC*GAACGCAGACAGAGCATGGG
STXBP2-7FS	**TGTAAAACGACGGCCAGT**CCGCAGTACCAGAAGGAGCT
STXBP2-7RS	*CAGGAAACAGCTATGACC*CCCTCCACCTCTCCACAAGC
STXBP2-8FS	**TGTAAAACGACGGCCAGT**CCTTGAGAGACCTGGTGCTGAG
STXBP2-8RS	*CAGGAAACAGCTATGACC*GTGGGAGACGCTGGCAAATG
STXBP2-9FS	**TGTAAAACGACGGCCAGT**CCAGGTTTCCCACTCTTGCTC
STXBP2-9RS	*CAGGAAACAGCTATGACC*GACCAGACCCGAAACACTGC
STXBP2-10FS	**TGTAAAACGACGGCCAGT**TCTGTGACCAGCCTCCTTCC

Table I. Continued.

Name of the primer	Sequence 5' to 3'
STXBP2-10RS	*CAGGAAACAGCTATGACC*CCTCAGCAGAGCAGATCGGT
STXBP2-11FS	**TGTAAAACGACGGCCAGT**CAGAGGCAGGAGGTGGAGATG
STXBP2-11RS	*CAGGAAACAGCTATGACC*TGTCCCTGTCCCTCAGCAAA
STXBP2-12FS	**TGTAAAACGACGGCCAGT**AAGTGGGAGGTGCTCATTGG
STXBP2-12RS	*CAGGAAACAGCTATGACC*AAGTCCAAGTTCTTAACCTCCATGA
STX11-1FS	**TGTAAAACGACGGCCAGT**TTGCCCACACCGAGGAATAC
STX11-1RS	*CAGGAAACAGCTATGACC*CTCGCTCAGCTCCTTCATGG
STX11-2FS	**TGTAAAACGACGGCCAGT**GCGAGGTCATCCACTGCAAG
STX11-2RS	*CAGGAAACAGCTATGACC*CTTTGGTGCGTCCTTCCCAG
PRF1-1FS	**TGTAAAACGACGGCCAGT**CCTTCCATGTGCCCTGATAA
PRF1-1RS	*CAGGAAACAGCTATGACC*GCCAGGATTGCAGTTTCTTC
PRF1-2FS	**TGTAAAACGACGGCCAGT**CCCTGGGTTCCAGTCCTAGT
PRF1-2RS	*CAGGAAACAGCTATGACC*GCCCTGTCCGTCAGGTACT
PRF1-3FS	**TGTAAAACGACGGCCAGT**CTGCACGTGCTGCTGGACA
PRF1-3RS	*CAGGAAACAGCTATGACC*CTGGTCCTTTCCAAGCTCAC
SH2D1A-1FS	**TGTAAAACGACGGCCAGT**GCTCGATCGAACCAAGCTAC
SH2D1A-1RS	*CAGGAAACAGCTATGACC*GGATTGAGGCGAAAGTGTGT
SH2D1A-2FS	**TGTAAAACGACGGCCAGT**TCTCACTGGAAACTGTGGTTGG
SH2D1A-2RS	*CAGGAAACAGCTATGACC*GCTAAACAGGACTGGGACCAAA
SH2D1A-3FS	**TGTAAAACGACGGCCAGT**ACTTCTCTTAGCATCCCTAGCAC
SH2D1A-3RS	*CAGGAAACAGCTATGACC*CTGGCTACATCTACTTTCTCACTGC
SH2D1A-4FS	**TGTAAAACGACGGCCAGT**AGGCTCAGGCATAAACTGAC
SH2D1A-4RS	*CAGGAAACAGCTATGACC*GCATTTGTAGCTCACCGAACTGT
XIAP-1FS	**TGTAAAACGACGGCCAGT**AGAATGTTTCTTAGCGGTCGTGTAG
XIAP-1RS	*CAGGAAACAGCTATGACC*GTTCCTCGGGTATATGGTGTCTGATAT
XIAP-2FS	**TGTAAAACGACGGCCAGT**TCTGGGAAGCAGAGATCATTTTG
XIAP-2RS	*CAGGAAACAGCTATGACC*CCTGGCATACTTGGGAAGCT
XIAP-3FS	**TGTAAAACGACGGCCAGT**AGTGTGTATTTCTTCCTCAAAGGATAA
XIAP-3RS	*CAGGAAACAGCTATGACC*CTCCCACTGCATGCTATCCAA
XIAP-4FS	**TGTAAAACGACGGCCAGT**CAGTGGGATAGGGAATTGGGTA
XIAP-4RS	*CAGGAAACAGCTATGACC*CACTGCCCAGCTAGCTCTCAT
XIAP-5FS	**TGTAAAACGACGGCCAGT**GGTGGCCAAGGCATCAGTAA
XIAP-5RS	*CAGGAAACAGCTATGACC*GCGCATCACAAGATCAGGAGT
XIAP-6FS	**TGTAAAACGACGGCCAGT**ACCCGCTCTGCTACAGAAAC
XIAP-6RS	*CAGGAAACAGCTATGACC*CACATCTGGCCCTTTCTTGCTTT
XIAP-7FSa	**TGTAAAACGACGGCCAGT**CAGATGCCACGGGTGAGTCA
XIAP-7RSa	*CAGGAAACAGCTATGACC*ATTGCCAACTAAAACACTGCCAT

The segment in bold font is a nonspecific tail named S1, which is added to the specific forward primers. The segment in italic font is a nonspecific tail named S2, which is added to the specific reverse primers. S1 and S2 are also used as sequencing primers.

In silico analysis of UNC13D c.2588G>A mutation. The UNC13D c.2588G>A/p.G863D mutation resulted in a substitution of the nonpolar and hydrophobic glycine (often involved in the formation of the turn structure) in the Munc13 homology domain 2 of protein UNC13D by the polar, and neutral aspartic acid (often involved in the formation of the coil structure). Multiple sequence alignment demonstrated that the amino acid at this position was highly conserved in available vertebrate species (Fig. 2A) and the alteration is predicted to be possibly damaging using PolyPhen-2 (Fig. 2B), and deleterious with SIFT *in silico* analysis. I-TASSER also demonstrated significant differences in the 3D structures of the wild-type and mutant-type proteins (Fig. 2).

Discussion

In 2005, Clementi *et al* (10) first reported that 8/29 (27.6%) unrelated Italian patients with lymphoma carried PRF1 mutations and 5 of them carried PRF1 c.272C>T/p.A91V heterozygous mutation. In 2014, Ciambotti *et al* (11) observed mutations in 23/84 (27.4%) Italian patients with anaplastic large cell lymphoma following genotype analysis of PRF1,

Table II. Gene mutations observed in 24 patients with lymphoma.

Author, name	Patient	Sex	Age at diagnosis, years	Diagnosis	Gene	Mutation	Genotype	(Refs.)
	P1	M	7	HL	UNC13D	c.514C>A/p.R172S	Het.	Novel observation
Tong *et al*, 2011; Zhang *et al*, 2014	P2	M	26	HL	UNC13D	c.1232G>A/p.R411Q	Het.	(12,20)
Sieni *et al*, 2011	P3	M	32	HL	UNC13D	c.1241G>T/p.R414L	Het.	(21)
	P4	M	17	B-NHL	UNC13D	c.1894G>T/p.D632Y	Het.	Novel observation
	P5	M	3	HL	UNC13D	c.2495C>T/p.A832V	Het.	Novel observation
Tong *et al*, 2011; Zhang *et al*, 2011	P6	F	35	B-NHL	UNC13D	c.2553+5C>G	Het.	(12,22)
Tong *et al*, 2011	P7	F	54	NK/T-NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P8	M	46	NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P9	F	12	NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P10	M	40	B-NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P11	F	30	NK/T-NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P12	M	28	NHL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
Tong *et al*, 2011	P13	M	9	HL	UNC13D	c.2588G>A/p.G863D	Hom.	(12)
Tong *et al*, 2011; Zhang *et al*, 2011	P14	M	38	HL	UNC13D	c.2240G>A/p.S747N	Het.	(12,22)
Tong *et al*, 2011; Zhang *et al*, 2011	P14	M	38	HL	UNC13D	c.2553+5C>G	Het.	(12,22)
Tong *et al*, 2011	P15	M	29	HL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
					UNC13D	c.3067C>T/p.R1023C	Het.	Novel observation
Tong *et al*, 2011	P16	M	12	HL	UNC13D	c.2588G>A/p.G863D	Het.	(12)
					UNC13D	c.518C>T/p.T173M	Het.	Novel observation
					UNC13D	c.977C>T/p.S326L	Het.	Novel observation
Zhang *et al*, 2011	P17	F	36	HL	PRF1	c.10C>T/p.R4C	Het.	(22)
Zhang *et al*, 2011	P18	M	10	HL	PRF1	c.98G>A/p.R33H	Het.	(22)
Lu *et al*, 2009	P19	F	34	NK/T-NHL	PRF1	c.503G>A/p.S168N	Hom.	(23)
Trizzino *et al*, 2008	P20	M	29	B-NHL	PRF1	c.1066C>T/p.R356W	Het.	(24)
Trizzino *et al*, 2008	P21	F	19	HL	PRF1	c.1349C>T/p.T450M	Het.	(24)
Zhang *et al*, 2011	P22	M	24	NK/T-NHL	PRF1	c.10C>T/p.R4C	Het.	(22)
Zhang *et al*, 2011	P22	M	24	NK/T-NHL	PRF1	c.98G>A/p.R33H	Het.	(22)
Tong *et al*, 2011	P23	M	56	NK/T-NHL	PRF1	c.65delC/p.P22Rfs*29	Het.	(12)
Lu *et al*, 2009	P23	M	56	NK/T-NHL	PRF1	c.503G>A/p.S168N	Het.	(23)
Tong *et al*, 2011	P24	M	15	HL	STX11	c.842T>G/p.F281C	Het.	(12)

Het., heterozygous; Hom., homozygous; UNC13D, unc-13 homolog D; PRF1, perforin; STX11, syntaxin 11; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; NK/T, natural killer/T-cell; B, B-cell; M, male; F, female.

Table III. Allele frequencies of PRF1 c.272T and UNC13D c.2588A among different populations.

	Allele frequencies	
Populations/samples	PRF1 c.272T	UNC13D c.2588A
1000G-all populations	0.0132 (66/5008)	0.0014 (7/5008)
1000G-CHB	0 (0/206)	0.0097 (2/206)
1000G-CHS	0.0048 (1/210)	0.0048 (1/210)
1000G-CDX	0 (0/186)	0.0108 (2/186)
1000G-JPT	0 (0/208)	0.0048 (1/208)
1000G-BEB	0 (0/172)	0.0058 (1/172)
1000G-FIN	0.0253 (5/198)	0 (0/198)
1000G-GBR	0.0385 (7/182)	0 (0/182)
1000G-TSI	0.0561 (12/214)	0 (0/214)
Patients in the present study	0 (0/180)	0.0556 (10/180)
Controls in the present study	0 (0/120)	0.0095 (4/420)

CHB, Han Chinese in Beijing China; CHS, Southern Han Chinese; CDX, Chinese Dai in Xishuangbanna, China; JPT, Japanese in Tokyo Japanese; BEB, Bengali from Bangladesh; FIN, Finnish in Finland; GBR, British in England and Scotland; TSI, Toscani in Italia; UNC13D, unc-13 homolog D; PRF1, perforin; 1000G, 1000 Genomes Project.

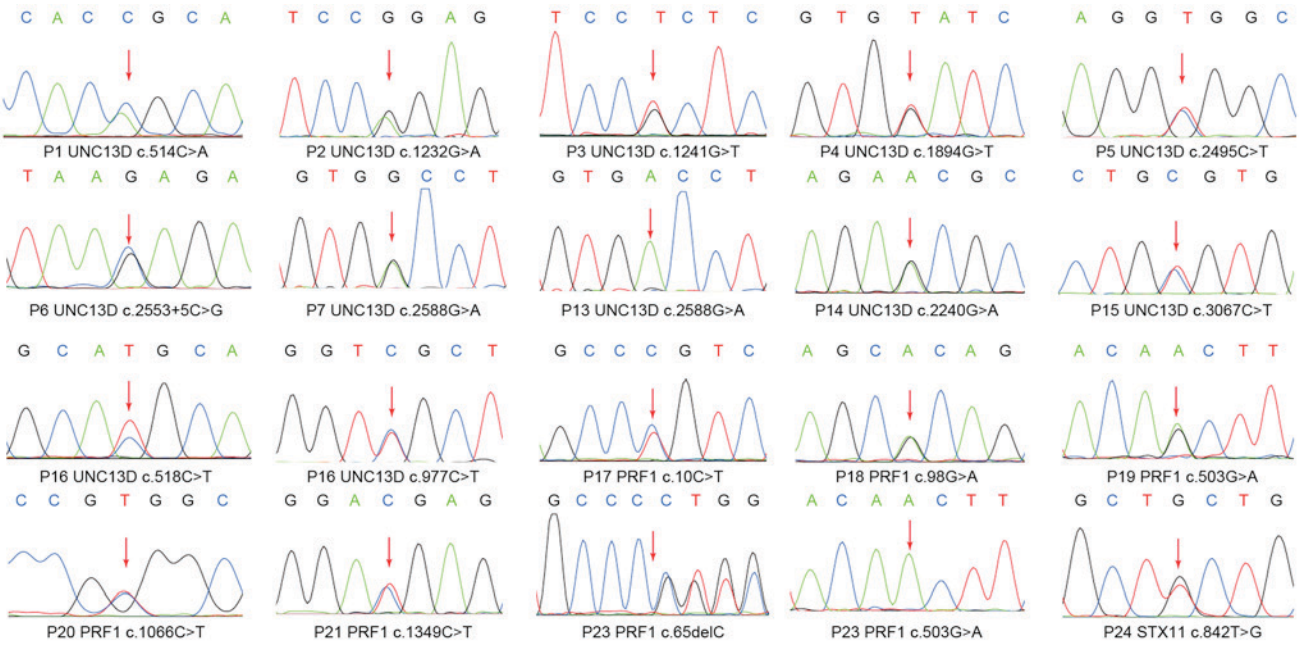

Figure 1. Sanger sequencing chromatogram of the genomic polymerase chain reaction product of the 24 patients with lymphoma. Red arrows indicate the mutations detected. UNC13D, unc-13 homolog D; PRF1, perforin; STX11, syntaxin 11; P, patient number.

UNC13D and SH2D1A. Twenty-one patients (25%) carried PRF1 mutations and the other 2 patients had mutations of UNC13D. PRF1 c.272C>T/p.A91V mutation was also the most common mutant genotype (11/84).

In the present study 6 genes, which are all involved in cytotoxic function of natural killer cells and cytotoxic T lymphocytes, were identified in 90 Chinese patients with lymphoma. The results demonstrated the association of germline defective mutations and development of lymphoma. The majority of mutations detected in the current study were heterozygous missense mutations, which were consistent with previous reports (10,11). This may explain why these patients developed lymphoma later in life rather than outbreak fatal FHL during infancy. Such monoallelic mutations may contribute to the pathogenesis of the disease, but are not sufficient to initiate the disease phenotype alone. Additional unidentified genetic defects, or possibly even environmental factors, may contribute to the development of lymphoma (10). What was different from reports in Europe was that the most common mutant gene in the present study was UNC13D while PRF1 was less frequently involved, indicating a distinct mutation spectrum in Chinese patients with lymphoma.

Notably, no hot spot region or predominant pathogenic mutation in UNC13D had been previously identified (18). In the current study; however, 9/16 UNC13D mutation

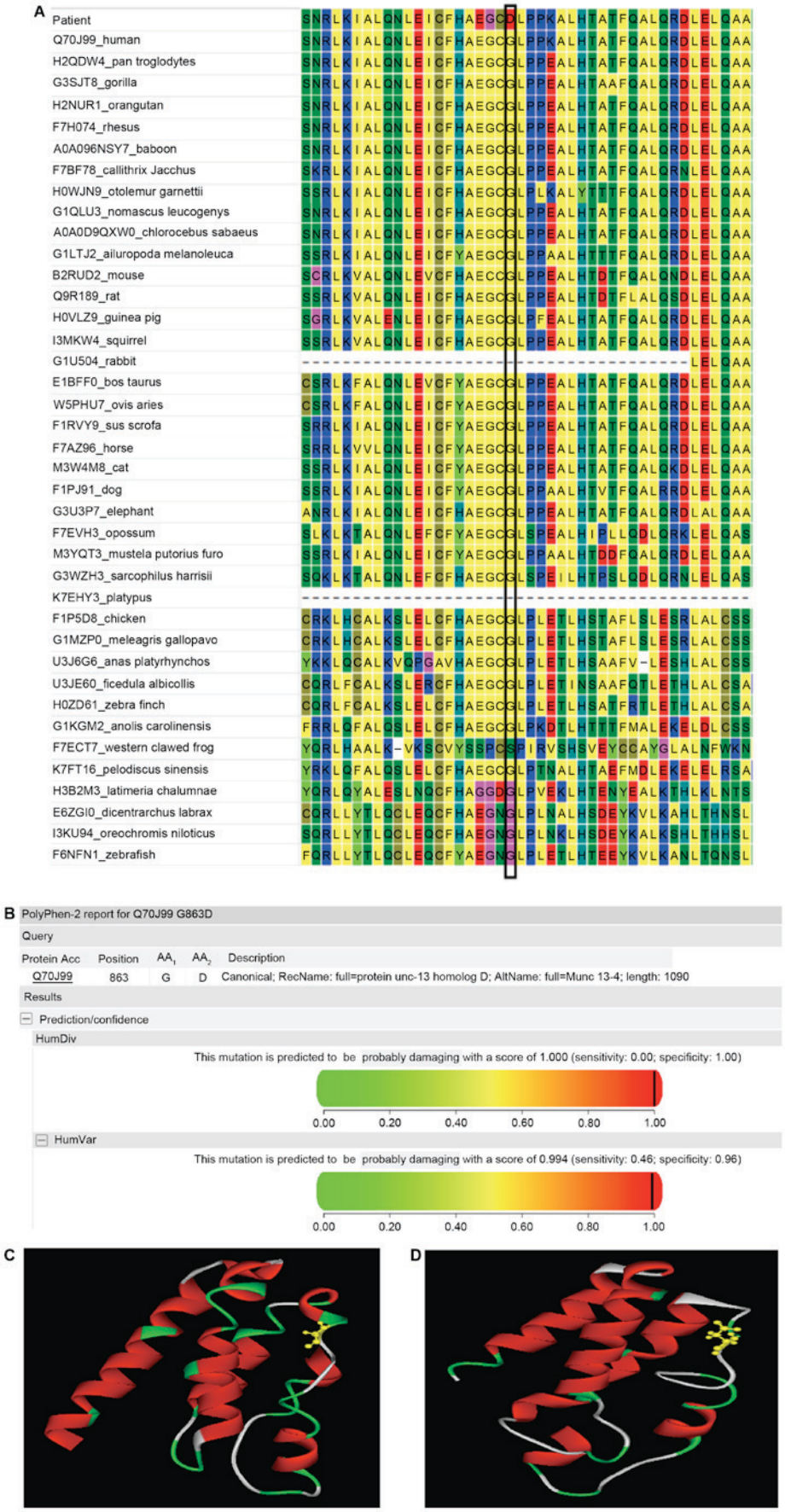

Figure 2. *In silico* analysis of UNC13D c.2588G>A mutation. (A) Multiple sequence alignment demonstrated that the amino acid at this position was highly conserved in available vertebrate species (Uniprot ID, species). (B) Polymorphism Phenotyping version 2.0 predicted that this mutation is possibly damaging with a score of 0.994. (C) The 3D structure of the wild-type UNC13-4 MHD2. The molecular in yellow is the 863th amino acid of the UNC13-4 protein. (D) 3D structure of the mutant-type UNC13-4 MHD2. The molecular in yellow is the 863th amino acid of the UNC13-4 protein. MHD2, Munc13 homology domain 2; UNC13D, unc-13 homolog D.

carriers exhibited c.2588G>A/p.G863D mutation, including 1 homozygous and 8 heterozygous. This single amino acid substitution occurred in an evolutionary conserved position and was predicted to be pathogenic using PolyPhen-2, SIFT, and I-TASSER. Furthermore, statistical analysis revealed that this mutation was significantly associated with the risk of developing lymphoma. In addition, none of our patient harbored the PRF1 c.272C>T/p.A91V mutation, which was most frequently reported in European populations (10,11). In the present consecutive cohort of >500 patients with diagnosed or suspected FHL, the PRF1 c.272C>T mutation was not identified (data not shown).

Data in the 1000 Genomes Project demonstrated that the allele frequency of PRF1 c272T was significantly higher in European population compared with that in Chinese and Japanese, supporting the concept of a Mediterranean origin of the mutation (11). However, the UNC13D c.2588A allele existed predominantly in Chinese, less in Japanese and Bengali, and was not identified in any other populations listed in this database (Table III). In regards to Korea, where UNC13D is the predominant causative gene in Korean patients with FHL, c.2588G>A was not reported (19). Collectively, the data obtained from the present study and the databases suggest that UNC13D c.2588G>A/p.G863D is a founder mutation of Chinese patients.

In conclusion, the current study provides a relatively comprehensive mutation spectrum of defective cytotoxicity associated genes in Chinese patients with lymphoma. Monoallelic germline mutations were identified to be most frequent in the present cohort, suggesting that partially impaired cytotoxic machinery may represent a predisposing factor for the development of lymphoma. In addition, UNC13D was identified as the predominant causative gene, while PRF1 was less frequently involved. Furthermore, UNC13D c.2588G>A/p.G863D, which is not reported in other populations, is a founder mutation in Chinese patients.

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